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Total number of authors:
29

Published in:
Cell Reports

Link to article, DOI:
[10.1016/j.celrep.2017.10.034](https://doi.org/10.1016/j.celrep.2017.10.034)

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):

Wewer Albrechtsen, N. J., Kuhre, R. E., Hornburg, D., Jensen, C. Z., Hornum, M., Dirksen, C., Svane, M., Gasbjerg, L. S., Jørgensen, N. B., Gabe, M. N., Balk-Møller, E., Albrechtsen, R., Winther-Sørensen, M., Galsgaard, K. D., Meissner, F., Jorsal, T., Lund, A., Vilsbøll, T., Eliassen, R., ... Holst, J. J. (2017). Circulating Glucagon 1-61 Regulates Blood Glucose by Increasing Insulin Secretion and Hepatic Glucose Production. *Cell Reports*, 21(6), 1452-1460. <https://doi.org/10.1016/j.celrep.2017.10.034>

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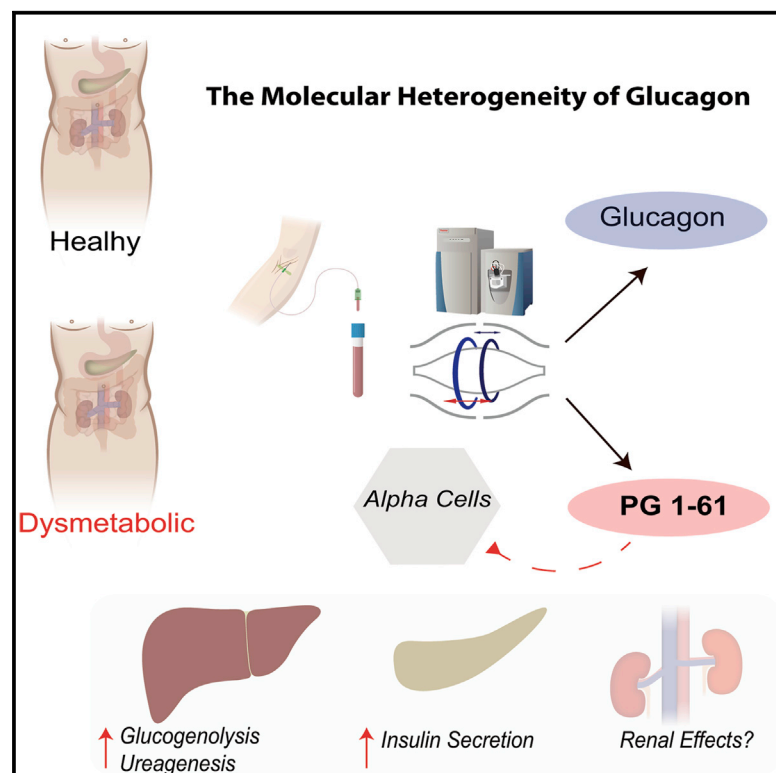
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Cell Reports

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Graphical Abstract



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In Brief

Wewer Albrechtsen et al. identify a glucagon-like molecule, PG 1-61, in humans using mass-spectrometry-based proteomics. PG 1-61 activates the glucagon receptor, stimulates insulin secretion, and activates key gluconeogenic enzymes. In dysmetabolic conditions, PG 1-61 is upregulated and may therefore serve as a marker of alpha cell stress.

Highlights

- PG 1-61 is a glucagon-like peptide in humans
- Hyperglucagonemia may include PG 1-61, as well as glucagon
- PG 1-61 stimulates insulin secretion and activates human hepatocytes
- PG 1-61 may be a surrogate marker of alpha cell dysfunction



Circulating Glucagon 1-61 Regulates Blood Glucose by Increasing Insulin Secretion and Hepatic Glucose Production

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<https://doi.org/10.1016/j.celrep.2017.10.034>

SUMMARY

Glucagon is secreted from pancreatic α cells, and hypersecretion (hyperglucagonemia) contributes to diabetic hyperglycemia. Molecular heterogeneity in hyperglucagonemia is poorly investigated. By screening human plasma using high-resolution-proteomics, we identified several glucagon variants, among which proglucagon 1-61 (PG 1-61) appears to be the most abundant form. PG 1-61 is secreted in subjects with obesity, both before and after gastric bypass surgery, with protein and fat as the main drivers for secretion before surgery, but glucose after. Studies in hepatocytes and in β cells demonstrated that PG 1-61 dose-dependently increases levels of cAMP, through the glucagon receptor, and increases insulin secretion and protein levels of enzymes regulating glycogenolysis and gluconeogenesis. In rats, PG 1-61 increases blood glucose and plasma insulin and decreases plasma levels of amino acids *in vivo*. We conclude that glucagon variants, such as PG 1-61, may contribute to glucose regulation by stimulating hepatic glucose production and insulin secretion.

INTRODUCTION

Glucagon is a peptide hormone secreted from pancreatic α cells in response to hypoglycemia and hyperaminoacidemia (Gromada et al., 2007). Hypersecretion of glucagon (hyperglucagonemia) is an important element of the pathophysiology of diabetes and contributes to its pathogenesis (Unger and Cherrington, 2012).

The glucagon gene (GCG), which encodes the precursor molecule of glucagon, proglucagon (PG), is expressed in pancreatic α cells, in enteroendocrine L cells and in the brain stem (Vrang and Larsen, 2010). The processing of PG is strictly regulated by the differential expression of the two prohormone convertases, 2 (PC2) and 1/3 (PC1), which, in α cells, gives rise to glucagon (because of PC2 activity), while in the L cells, which express PC1, a number of “glucagon-like” hormones, including the incretin hormone glucagon-like peptide-1 (GLP-1) are formed (Figure 1A). Heterogeneity of the products of PG (in particular, PC2-dependent processing) has previously been reported in patients with glucagon-producing tumors (Holst, 1983) (glucagonoma) and in uremic pigs (Baldiessa and Holst, 1986). However, the extent to which molecular heterogeneity is normally seen and whether it is involved in diabetes and/or other metabolically related diseases are unknown (Unger and Cherrington, 2012).

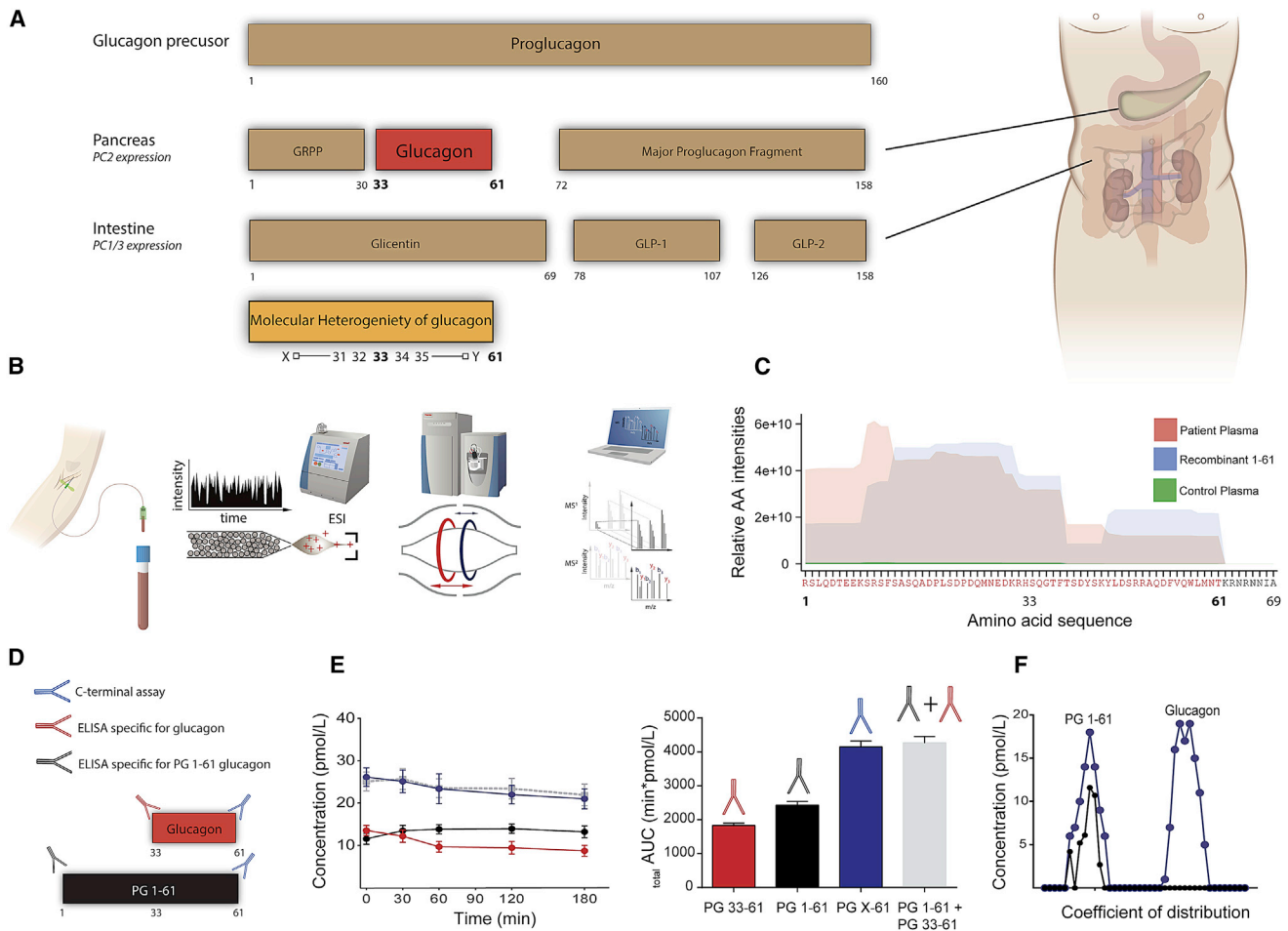


Figure 1. PG 1-61, a Glucagon Variant, Identified in Human Plasma by Mass Spectrometry

(A) Overview of the processing of proglucagon (1-160). In the pancreas, proglucagon is processed by prohormone convertase 2 (PC2), resulting in the formation of glucinotin-related pancreatic polypeptide (GRPP), glucagon, and the major proglucagon fragment. In the intestine, the actions of prohormone convertase 1/3 (PC1/3) lead to the formation of glucinotin, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2). Below, potential (denoted as X and Y) N-terminally elongated and C-terminally truncated forms of glucagon are depicted.

(B) A mass-spectrometry-based platform for identification of low-abundant peptides such as glucagon. In short, blood is taken from a subject, and the plasma is subjected to ultra-pressure liquid chromatography (UPLC), and the peptides are sprayed into an Orbitrap-based mass spectrometer, using an electrospray technique (ESI). The identified spectra are deconvoluted into amino acid sequences using the MaxQuant software package.

(C) Separate plasma pools, obtained from subjects with kidney failure ($n = 8$) and from healthy subjects ($n = 8$), were subjected to the platform shown in (B), and the corresponding amino acid intensities are depicted as red (kidney failure) and green (healthy subjects). Synthesized PG 1-61 (positive control) is depicted in blue.

(D) By comparing plasma levels of immunoreactive total glucagon (i.e., PG 1-61 + PG 33-61 [glucagon]), using a C-terminal assay (blue), to plasma levels of PG 1-61 (black) and glucagon 33-61 (red) using two sandwich ELISAs, we were able to verify immunoreactive PG 1-61 in plasma in response to an oral glucose load

(E) in the same kidney failure individuals used in our mass-spectrometry-based platform (C).

(F) Size-exclusion chromatography identified two major immunoreactive glucagon-like moieties using the C-terminal assay (blue): one identified having similar coefficient of distribution (K_d) as recombinant PG 1-61 ($K_d = 0.22$) (only immunoreactive peak identified using the PG 1-61 assay, black curve) and one similar to pancreatic glucagon (33-61) ($K_d = 0.8$).

In the present study, we investigated the molecular heterogeneity of PG products in conditions known to be associated with hyperglucagonemia, such as post gastric bypass, type 2 diabetes, and renal failure. An N-terminally elongated glucagon molecule, PG 1-61, was identified using a mass spectrometry-based proteomic platform. Furthermore, using an array of *in vitro* and *in vivo* studies, we demonstrate that PG 1-61 possesses gluco-regulatory effects through activation of the glucagon receptor expressed in pancreatic β cells and hepatocytes.

RESULTS

PG 1-61 Identified in Human Plasma by Mass Spectrometry, Immunoassays, and Size-Exclusion Chromatography

We identified an N-terminally elongated glucagon molecule using a validated and unbiased mass-spectrometry-based platform (Figures 1B and 1C), corresponding to the amino acid sequence of PG 1-61 (RSLQDTEEKSRFSASQADPLSDPDQMNEKDRHS

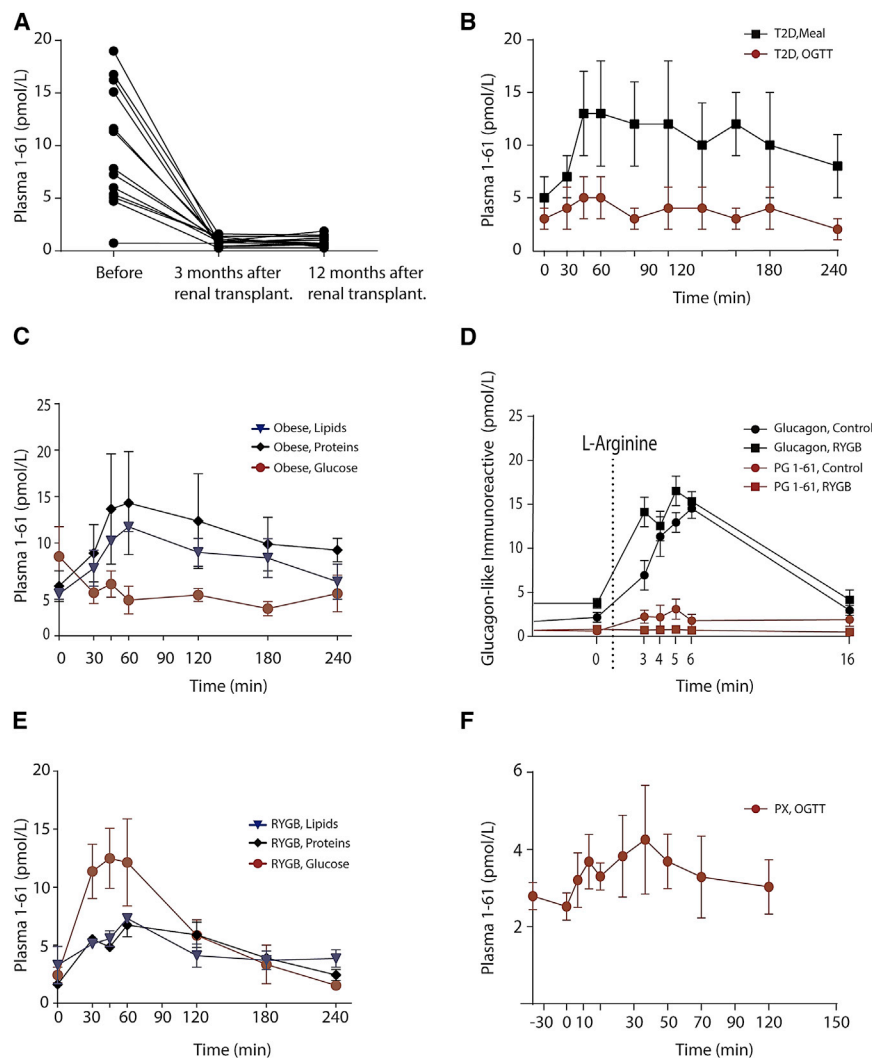


Figure 2. PG 1-61 Responses in Subjects with a Variety of Clinical Conditions Characterized with Hyperglucagonemia

(A) Plasma levels of PG 1-61 during an oral glucose tolerance test (0-min time point was analyzed and is shown) in subjects with kidney failure (end-stage renal disease) before, 3 months, and 12 months after renal transplantation.

(B) PG 1-61 responses during a meal stimulation test (black) and an oral glucose tolerance test (OGTT) (red) in subjects with type 2 diabetes (T2D). (C) PG 1-61 responses during an oral glucose load (red), fat ingestion (blue), or protein ingestion (black) in obese subjects.

(D) PG 1-61 (red) and glucagon (black) responses during an L-arginine test in healthy subjects (circles) or in gastric-bypass-operated subjects (squares) (Roux-en-Y gastric bypass).

(E) PG 1-61 responses during an oral glucose tolerance test (red), fat ingestion (blue), or protein ingestion (black) in gastric-bypass-operated subjects (Roux-en-Y gastric bypass).

(F) PG 1-61 responses during an oral glucose tolerance test in fully pancreatectomized subjects (PX). $n = 8-12$.

QGTFTSDYSKYLDSSRAQDFVQWLMNT), in plasma from patients with hyperglucagonemia due to kidney failure (end-stage renal disease [ESRD]) but *not* in plasma from healthy controls. Furthermore, we identified several other N-terminally elongated glucagon variants, but their abundancies were much less (10-fold) compared to those of PG 1-61 (Figure S1).

The structure of the N-terminally elongated glucagon variant was consistent with its reactions toward two sandwich ELISAs: lack of reactivity in an assay specific for fully processed glucagon and full reactivity in an assay designed for reaction with PG 1-61, as well as in a validated C-terminal-specific glucagon radioimmunoassay (Figure 1D). Secretory profiles of glucagon-like immunoreactivity assessed by a C-terminal assay were almost superimposable to a profile calculated as the sum of fully processed glucagon and PG 1-61 concentrations (Figure 1E) ($AUC_{0-180min}$ for the C-terminal assay = $4,021 \pm 195 \text{ min} \times \text{pM}$, compared to $AUC_{0-180min}$ for the two sandwich ELISAs: $4,102 \pm 219 \text{ min} \times \text{pM}$, $p = 0.74$). Finally, size-exclusion chromatography of pooled plasma, from the same kidney failure patients as described above, with concomitant measurements with a C-terminal glucagon assay and a PG 1-61 spe-

cific assay, identified a peak ($K_d = 0.28$) corresponding to that of exogenous PG 1-61 (Figure 1F), thereby independently verifying the results obtained using the mass-spectrometry-based platform.

PG 1-61 Is Secreted in Several Clinical Conditions Associated with Hyperglucagonemia

Thirteen out of 14 patients with renal dysfunction had measurable plasma levels of PG 1-61, which dropped significantly ($p < 0.001$) 3 months after renal

transplantation (in parallel with normalization of the kidney function), and remained stable at a 12-month follow-up (Figure 2A). There were no differences in plasma levels of PG 1-61 in kidney failure patients with diabetes compared to those with normal glucose tolerance (7 in total). To investigate whether immunoreactive PG 1-61 is only found in subjects with insufficient renal plasma clearance (end-stage renal disease), we analyzed plasma from subjects with other pathologies associated with hyperglucagonemia, namely, type 2 diabetes and morbid obesity.

No detectable PG 1-61 was found in plasma from patients with type 2 diabetes during an oral glucose tolerance test (OGTT) (concentrations remained around the detection limit of the sandwich ELISA $\sim 1 \text{ pM}$) (Figure 2B), whereas measurable levels were found during a meal stimulation test ($nAUC_{0-240min}$, OGTT $182 \pm 90 \text{ min} \times \text{pM}$, compared to meal $AUC_{0-240min}$, meal stimulation $1,430 \pm 284 \text{ min} \times \text{pM}$, respectively, $p < 0.01$). In obese subjects, protein and fat ingestion increased plasma levels of PG 1-61 (Figure 2C). In contrast, glucose ingestion was not associated with any rise in PG 1-61 levels, and, if anything, there was a trend for levels to be reduced. Extracts of gut biopsies (mucosa)

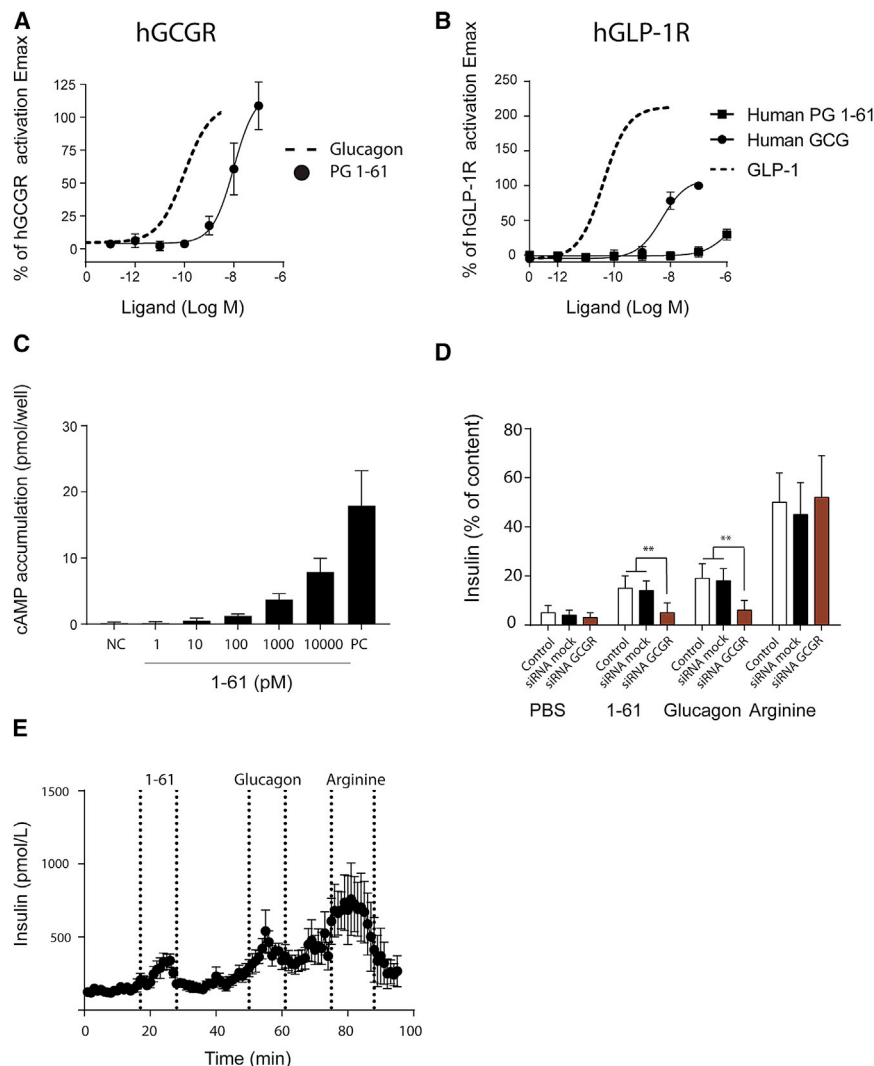


Figure 3. PG 1-61 Activates GCGR and Regulates Blood Glucose by Stimulating Insulin Secretion from Cultured β Cells and Isolated Pancreases

(A and B) PG 1-61 activated the human glucagon receptor (hGCGR) (A) but not the human glucagon-like peptide 1 receptor (hGLP-1R) (B) with same efficacy but with lower potency compared to native glucagon in transiently transfected COS-7 cells expressing hGCGR (A) and hGLP-1R (B).

(C) PG 1-61 dose-dependently stimulated levels of cAMP in INS1 cells. Positive control (PC), consisting of Bombesin, Forskolin, and IBMX, is shown.

(D) PG 1-61 (1 nM) stimulated the secretion of insulin in INS1 cells; thus, this could be significantly reduced by small interfering RNA of the glucagon receptor (red), compared to small interfering RNA control (black). PBS was used as negative control, L-arginine was used as a positive control, and glucagon was used as comparator to the effects of PG 1-61.

(E) Perfusion of isolated rat pancreases ($n = 6$) with PG 1-61 (1 nM), glucagon (1 nM), and L-arginine as positive control. PG 1-61 significantly ($***p < 0.001$) increased insulin secretion. $n = 6-8$.

tomized patients, where the surgery results in similar anatomical rearrangement of the gastrointestinal tract as after Roux-en-Y gastric bypass surgery, an oral glucose load led to modest increases in PG 1-61 plasma levels (Figure 2F).

PG 1-61 Activates the Glucagon Receptor, Stimulates Insulin Secretion in β Cells, and Increases Blood Glucose in Rats

In transiently transfected COS-7 cells, synthetic PG 1-61 (synthesized according to the sequence identified in humans)

activated the human glucagon receptor (hGCGR) as a full agonist with a 100-fold decreased potency compared to native glucagon ($\text{LogEC}_{50, \text{PG 1-61}} = -8.0 \pm 0.22$ compared to $\text{LogEC}_{50, \text{glucagon}} = -10.0 \pm 0.17$) (Figure 3A). In contrast, it acted with very low potency on cells expressing hGLP-1R and only reached 38% of E_{max} of GLP-1 at a concentration of 1 μM , whereas glucagon acted as a partial agonist with 100-fold lower potency and an E_{max} of 78% compared to native GLP-1 ($\text{LogEC}_{50, \text{PG 1-61}} < -6$, compared to $\text{LogEC}_{50, \text{glucagon}} = -8.2 \pm 0.08$ and $\text{LogEC}_{50, \text{GLP-1}} = -10.2 \pm 0.11$) (Figure 3B).

PG 1-61 dose-dependently increased accumulation of cAMP in cultured rat β cells (INS1) (Figure 3C) and significantly stimulated insulin release compared to PBS administration alone (Figure 3D, $p < 0.001$). The effect was comparable to that of native glucagon. In INS1 cells with small interfering RNA (siRNA)-induced knockdown of the rat glucagon receptor (rGCGR) knockdown estimated to $\sim 70\%$ by qPCR, data not shown, PG 1-61-induced insulin secretion was significantly blunted compared to small interfering RNA mock-treated cells

from obese subjects did not contain immunoreactive PG 1-61 (< 1 pmol/g) but had high levels of other peptides derived from intestinal processing of proglucagon (GLP-1, 58 ± 12 pmol/g; glicentin, 102 ± 23 pmol/g; oxyntomodulin, 32 ± 6 pmol/g), all three derived from the proglucagon-expressing L cells. Furthermore, extracts from three human healthy pancreases showed detectable levels of PG 1-61 (above limits of detection), although compared to glucagon this was rather low (PG 1-61: 2 pmol/g, glucagon 29 pmol/mg). Collectively, suggesting that PG 1-61 in some conditions may be derived from pancreatic α cells and co-secreted with pancreatic glucagon.

In both Roux-en-Y gastric bypass (RYGB)-operated and BMI-matched obese individuals, i.v. administration of L-arginine (an α cell secretagogue) robustly stimulated glucagon secretion (Figure 2D). PG 1-61 levels were also increased in the obese controls, albeit to a much lower extent than glucagon, but remained undetectable in the gastric bypass subjects (Figure 2D). Furthermore, glucose, but not fat and protein, elicited a secretory PG 1-61 response in gastric bypass individuals (Figure 2E). In pancreatec-

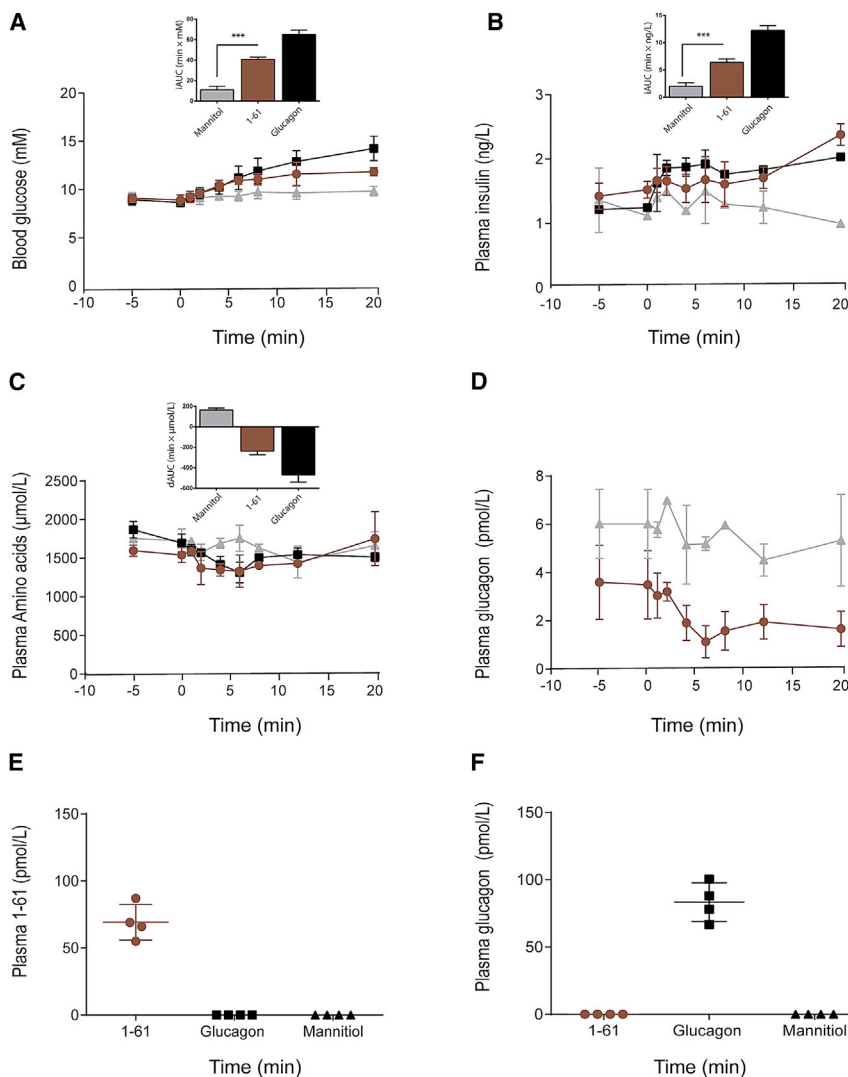


Figure 4. PG 1-61 Regulates Blood Glucose in Rats by Stimulating Insulin Secretion

Intravenous injection with mannitol (1 g/kg, gray), PG 1-61 (1 pmol, red), or glucagon (1 pmol, black) in rats. Mannitol was used as a control for volume and osmolarity. Plasma samples were obtained subsequently during a 20-min period.

(A and B) Both blood glucose (A) and plasma insulin levels (B) increased significantly after the injection of PG 1-61 and glucagon, respectively.

(C) Plasma amino acids decreased significantly after the injection of PG 1-61 and glucagon.

(D) Plasma glucagon levels decreased significantly after injection of PG 1-61 (red) but not mannitol (gray).

(E and F) Plasma levels of PG 1-61 (E) in PG 1-61, glucagon, and mannitol-treated rats are shown, and plasma levels of glucagon (F) in PG 1-61, glucagon, and mannitol-treated rats are shown. $n = 4-8$.

was not detected in the PG 1-61-treated rats indicates that PG 1-61 is not cleaved in the plasma to form glucagon. Administration of PG 1-61 to diabetic animal models did, however, not seem to inhibit the secretion of glucagon (Figure S2).

PG 1-61 Increases cAMP Levels in Primary Human Hepatocytes and Increases the Expression of Enzymes Related to Gluconeogenesis in the HEP1 Cell Line in a Glucagon Receptor-Dependent Manner

PG 1-61 dose-dependently increased intracellular levels of cAMP in primary human hepatocytes (HMC510) (Figure 5A) and in cultured human hepatocytes (HepG2) (Figure 5B). Small interfering

(Figure 3D, $p < 0.01$). Perfusion of isolated rat pancreases with PG 1-61 (1 nM) resulted in significant increases in insulin secretion compared to baseline ($tAUC_{1-61}$: $3,534 \pm 321$ min \times pM versus $tAUC_{baseline}$: $2,073 \pm 521$ min \times pM, $p < 0.001$) (Figure 3E).

i.v. administration of PG 1-61 (1 nM) in rats resulted in a rapid and robust increase in blood glucose compared to mannitol-treated rats ($tAUC_{0-20min}$: 41 ± 9 min \times mM versus 11 ± 10 min \times mM, $p < 0.001$) (Figure 4A). Plasma insulin increased transiently in response to both PG 1-61 and glucagon administration, but not mannitol (Figure 4B), and the summed molarity of amino acids (total plasma levels of amino acids) decreased (Figure 4C). PG 1-61 inhibited the secretion of pancreatic glucagon measured using a highly specific sandwich ELISA (Figure 4D) (which does not react with PG 1-61, unlike “traditional” (C-terminally directed) glucagon assays). Analysis of plasma using the two specific sandwich ELISAs for PG 1-61 and pancreatic glucagon, respectively, confirmed their specificity (i.e., no measurable PG1-61 in rats receiving exogenous glucagon and vice versa [Figures 4E and 4F]). Moreover, the observation that immunoreactive glucagon

RNA knockdown of hGCGR (knockdown estimated to $\sim 75\%$ by qPCR, Figure S3) attenuated both PG 1-61 and glucagon-stimulated increases in cAMP compared to small interfering RNA-treated controls in cultured hepatocytes (Figure 5B). 30-min incubation with PG 1-61 significantly increased protein levels of the enzymes protein kinase A (PKA), glucose-6-phosphatase (G6P), phosphorylase kinase (PKC-1), glycogen phosphorylase (GP), and phosphoenolpyruvate carboxykinase (PECK), essential for the regulation of gluconeogenesis and glycogenolysis (Figure 5C). In small interfering RNA-treated cells, the protein levels of these enzymes tended to be lower. Incubation for 3 hr with PG 1-61 showed more pronounced effects on the protein levels of protein kinase A, G6P, PKC-1, GP, and PECK (Figure 5D). Corresponding western blots are shown in Figures S3, S4, and S5.

DISCUSSION

Here, we unequivocally identified a glucagon variant (PG 1-61) in humans, using a mass-spectrometry-based platform for

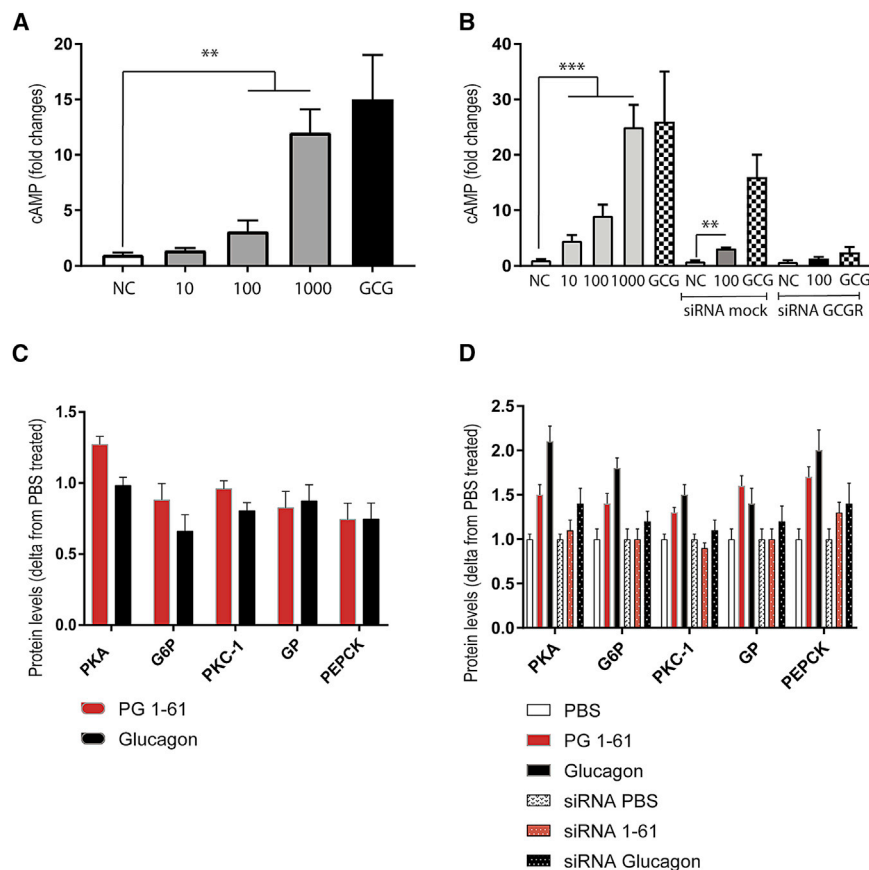


Figure 5. PG 1-61 Stimulates cAMP Production in Primary and Cultured Human Hepatocytes and Increases Protein Content of Enzymes Related to Gluconeogenesis and Glycogenolysis

(A) PG 1-61 stimulated cAMP production dose-dependently (10–1,000 pM) in primary human hepatocytes. As negative control (NC), we used PBS, and, as positive control (GCG), we used 100 pM glucagon.

(B) PG 1-61 stimulated cAMP production dose-dependently (10–1,000 pM) in cultured human hepatocytes (HepG2). Small interfering RNA mock and small interfering RNA glucagon receptor (siRNA GCGR)-mediated knockdown are shown.

(C) PG1-61 (red, 1,000 pM) increased protein levels (30 min of incubation) of glucose-6-phosphatase (G6P), phosphorylase kinase (PKC-1), glycogen phosphorylase (GP), and phosphoenolpyruvate carboxykinase (PECK). Administration of glucagon (1,000 pM) is shown in black.

(D) PG1-61 (red) increased protein levels (3-hr incubation) of protein kinase A (PKA), glucose-6-phosphatase (G6P), phosphorylase kinase (PKC-1), glycogen phosphorylase (GP), and phosphoenolpyruvate carboxykinase (PECK). In small interfering RNA-treated cells, the protein levels of these enzymes were not significantly different from PBS-treated cells (white). Corresponding original western blots are shown in Figure S4, and uncropped blots are shown in Figures S5 and S6. **p < 0.01, ***p < 0.001 tested using one-way ANOVA corrected for multiple testing (Sidak-Holm). n = 3–6.

low-abundant plasma peptides (Wewer Albrechtsen et al., 2016), and subsequently characterized its potential biological functions *in vitro*, *ex vivo*, and *in vivo*. Collectively, our data suggest that circulating PG 1-61 may contribute to the dysregulation of glucose homeostasis in clinical conditions including renal dysfunction, obesity, type 2 diabetes, and after Roux-en-Y gastric bypass, whereas its circulating levels in healthy lean controls did not exceed our analytical detection limit.

First, we demonstrated that PG 1-61 activates the hGCGR, but *not* the hGLP-1R, with similar efficacy as native glucagon but with lower potency. PG 1-61, like glucagon, increased cAMP and insulin secretion in cultured β cells mediated through GCGR signaling, as evidenced by the reduced effect seen after small interfering RNA induced knockdown of GCGR. A similar effect of PG 1-61 on insulin secretion was also observed in isolated rat pancreases, and, when it was given *in vivo* (intravenously, aimed at reaching similar concentrations to those used in the *ex vivo* experiments), blood glucose increased and plasma levels of amino acids decreased. The liver is the primary target for glucagon, and, accordingly, PG 1-61 also had hepatic effects. In primary hepatocytes expressing GCGR, PG 1-61 increased levels of cAMP, and this may be associated with relatively small changes in protein levels of enzymes related to the processes of glycogenolysis and gluconeogenesis. However, the latter need to be confirmed in future studies.

Our findings and previous observations (Holst, 1983, Challis et al., 2015, Baldissera and Holst, 1986, Rouillé et al., 1994, Friis-Hansen et al., 2001) suggest that a residual plasticity in proglucagon-producing cells may exist. A single study (Holst, 1983), comparing the gel filtration profiles of plasma from healthy controls to patients with glucagon-producing tumors, reported glucagon-like immunoreactivity with an estimated molecular size of 8,000 Da; however, that variant may actually represent another proglucagon-derived peptide, glicentin (PG 1–69, ~8,100 Da), as it contains the entire sequence of glucagon and reacts with antisera directed toward the glucagon sequence.

Using an unbiased mass-spectrometry-based platform, we identified a peptide having the same amino acid sequence as the first 61 amino acids of the proglucagon molecule. Mass-spectrometry technologies can be superior to immune-based assays as they have an absolute specificity (Aebbersold and Mann, 2016), but they can be very laborious. We therefore (together with Mercodia) developed an ELISA specific for PG 1-61 in humans, the accuracy of which was validated using the same mass-spectrometry platform as well as gel filtration chromatography. This sandwich ELISA allowed us to obtain secretory profiles of PG 1-61 in subjects with hyperglucagonemia of differing etiologies.

In a previous study in subjects with end-stage renal disease, we found that immunoreactive glucagon concentrations differed

according to the immunoassay employed (higher using a C-terminally directed glucagon assay compared to a sandwich ELISA combining N- and C-terminal antibodies) (Wewer Albrechtsen et al., 2014), indicating that the apparent hyperglucagonemia was not due to elevated levels of fully processed glucagon. Analyzing plasma samples from these subjects in the current study established PG 1-61 as the major circulating glucagon variant, with levels increasing in response to an oral glucose load. However, after kidney transplantation, levels of immunoreactive PG 1-61 were dramatically reduced, suggesting that the increased levels observed before transplantation likely reflect decreased renal clearance of this component rather than adaptive changes in proglucagon producing cells leading to increased secretion. On the other hand, given the complete absence of PG 1-61 in the plasma from healthy subjects, it cannot be fully excluded that the presence of this component in the subjects with renal insufficiency might be associated specifically with some aspect of the pathology of their disease. In addition, as glucagon may be affected by freeze-thaw cycles (Wewer Albrechtsen et al., 2015) the same could be true for PG 1-61, and, as such, we may therefore underestimate plasma concentrations of PG 1-61. PG 1-61 could also have a direct effect on the kidneys and thereby contribute to regulate gluconeogenesis; however, further studies will be needed to explore these possibilities.

PG 1-61 was not detected in plasma from subjects with type 2 diabetes during oral glucose administration, but a meal challenge did lead to an increase in plasma levels. Moreover, in morbidly obese subjects and in gastric-bypass-operated individuals, plasma levels of PG 1-61 increased in response to oral administration of macronutrients, but, intriguingly, the magnitude of the responses to the different stimuli varied between the two groups of subjects. In obese subjects, both protein and fat led to increases in immunoreactive PG 1-61, whereas glucose was without effect (as was also seen in subjects with type 2 diabetes). In contrast, in the gastric-bypass-operated individuals, glucose seems to constitute the major driver for PG 1-61 secretion, with only minor effects of protein and fat. These observations raise questions, not only about the mechanisms leading to PG 1-61 secretion, but also about its origin: in the gut or the pancreas? In support of gut-derived PG 1-61, the anatomical rearrangements associated with total pancreatectomy also lead to glucose-induced PG 1-61 secretion. As such, it seems that the molecular heterogeneity of hyperglucagonemia may be derived either from the pancreatic α cells in subjects with obesity and or type 2 diabetes, or from enteroendocrine L cells scattered along the small intestine in patients with rearrangement of such anatomical compartments. The residual plasticity, activated upon metabolic or anatomical alternations, in both α cells and L cells may therefore reflect a potential path to understand and to target dysmetabolic conditions.

Plasma concentrations of PG 1-61 were relatively low (picomolar range), which may be related to the instability of the molecule, as we previously shown for glucagon (Wewer Albrechtsen et al., 2015), and, since it also had lower potency on the glucagon receptor compared to the native peptide, one may question its relevance to dysregulated glucose metabolism in diabetes. That said, PG 1-61 did have glucose regulatory capabilities in human hepatocytes and did significantly increase blood glucose levels

in the *in vivo* rat studies, supporting a role for PG 1-61 in glucose metabolism at least under these circumstances. PG 1-61 may be subject to further cleavage, thereby potentially generating increases in plasma glucagon concentration. However, i.v. injection in rats was not associated with increases in plasma glucagon levels—rather glucagon tended to decrease, which might reflect a negative feedback mechanism involving the interaction with the glucagon receptor on pancreatic α cells. However, in diabetic animal models, PG 1-61 did not inhibit secretion of glucagon, suggesting that such a negative feedback circuit may be blunted in diabetic animals, as a consequence of their hyperglycemia.

In conclusion, we have demonstrated that molecular heterogeneity of glucagon exists, with PG 1-61 being a major additional component. Hyperglucagonemia may therefore, not only reflect hypersecretion of fully processed glucagon, but also of PG 1-61. Moreover, given that PG 1-61 can increase hepatic glucose production and insulin secretion, at least in *in vitro* and animal studies, it may influence glucose homeostasis in a variety of metabolically related conditions, which are associated with hyperglucagonemia.

EXPERIMENTAL PROCEDURES

Approvals

The clinical studies were conducted according to the latest revision of the Helsinki Declaration, approved by the Scientific-Ethical Committee of the Capital Region of Denmark (H-C-2009-007, H-1-2012-123, H-1-2013-063) and/or the regional ethics committee (KF-01279825) and registered by the Danish Data Protection Agency (2016-41-4892, 2007-58-0015), and at [ClinicalTrials.gov](https://www.clinicaltrials.gov) (NCT01327378, NCT02372526, NCT02006459, NCT02161666). All participants gave informed written consent. Animal studies were conducted with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and approved by the local ethical committee (P-15-335, P15-369, P-14-213, and P 17-266).

Study Design

Reserve plasma samples, collected during an oral glucose tolerance test, from individuals with end-stage renal disease and matched, healthy controls were obtained from a comparative, observational study as described previously (Idorn et al., 2013). These samples were used for mass-spectrometry and size-exclusion chromatography and ELISA measurements. Reserve plasma samples from individuals with end-stage renal disease; fasting plasma and during an oral glucose test from 14 individuals, including nine with normal glucose tolerance, were obtained before and after kidney transplantation as part of a prospective, observational, national multicenter study including 54 of 81 (67%) patients, mostly Caucasians, with scheduled living donor kidney transplantation in the period between January 2006 and March 2008, as described previously (Hornum et al., 2010).

Reserve plasma samples from Roux-en-Y gastric bypass-operated subjects, BMI-matched obese individuals (Dirksen et al., 2016; Z.C. Jensen et al., 2016, Diabetes, conference) and subjects with type 2 diabetes (Baranov et al., 2016, Jørgensen et al., 2012) were obtained from previous studies. Participants underwent oral glucose tolerance test or meal stimulation with isocaloric, isovolemic meals of either glucose, fat, or protein (Z.C. Jensen et al., 2016, Diabetes, conference); in addition, we performed L-arginine stimulation tests (Dirksen et al., 2016). Finally, plasma from a previously published study in pancreatectomized patients during an oral glucose tolerance test was analyzed (Lund et al., 2016). Gut biopsies from obese individuals were from a previously published study (Rhee et al., 2015).

Animal Studies

Male Wistar rats (~250 g, from Taconic Ejby) were housed two per cage under standard conditions for at least 1 week before experiments. Experiments were

carried out on non-fasted rats immediately before their nocturnal feeding period (1,700 hr). Rats were divided into three weight-matched treatment groups (glucagon: 265 ± 2 g; PG 1-61: 264 ± 3 g; and D-mannitol: 264 ± 3 g, $n = 6/\text{group}$) and received an intravenous (i.v.) bolus of test substance dissolved in 0.9% NaCl so that the injection volume was 1.5 mL/300 g body weight (w/v, D-mannitol: 20.2%); rats from the same cage received different treatments.

For further details, see [Supplemental Experimental Procedures](#).

Isolated Perfused Rat Pancreas

See [Supplemental Experimental Procedures](#).

Cell-Culture Experiments

INS-1E cells were kindly provided by Professor Jens Højris Nielsen (University of Copenhagen, Denmark). Cells were grown in DMEM (cat. no. 31966-021, GIBCO) containing 5,000 U/mL Pen-Strep (cat. no. 15140-122, GIBCO) and 10% fetal bovine serum (FBS) (cat. no. Sv3016003, Thermo Scientific) before being seeded in 24-well plates (Nunc, Thermo Scientific) at a cell density of 4×10^4 per well. Primary human hepatocytes were obtained using CellStream Isolation Technology (cat. no. HMCS10, lot. no. HUE50-F, Thermo Scientific).

See [Supplemental Experimental Procedures](#).

Receptor Studies

See [Supplemental Experimental Procedures](#).

qPCR and Small Interfering RNAs

See [Supplemental Experimental Procedures](#).

Western Blot Analysis

See [Supplemental Experimental Procedures](#).

Peptides and Antibodies

See [Supplemental Experimental Procedures](#).

Biochemical Measurements

Glucagon levels were measured using a previously validated sandwich ELISA (Mercodia; cat. no. 10-1281/1271 [Wewer Albrechtsen et al., 2014]) employing an N-terminal anti-glucagon antibody as capture antibody and a C-terminal-specific anti-glucagon antibody as detection antibody. Levels of N-terminally elongated glucagon (PG 1-61) were measured using a sandwich ELISA (Mercodia, presently not commercially available), employing an N-terminal antibody specific for the N-terminal region of the proglucagon molecule (amino acid sequence RSLQDTEE) and the same C-terminal specific anti-glucagon antibody as detection antibody (see above). Insulin levels were measured using a sandwich ELISA from Mercodia (cat. no. 10-1250). cAMP was measured according to manufacturer's protocol as described above.

Gel Filtration of Human Plasma

Further details are described in [Supplemental Experimental Procedures](#) and elsewhere (Challis et al., 2015).

Mass-Spectrometry-Based Detection of Glucagon

Plasma samples were analyzed using a mass-spectrometry-driven platform (Wewer Albrechtsen et al., 2016), with antibody-independent prefractionation steps providing unbiased estimation of the various glucagon forms. See [Supplemental Experimental Procedures](#).

Data and Materials Availability

The accession number for the mass spectrometry proteomics data reported in this paper is ProteomeXchange Consortium via the PRIDE partner repository: PXD005677.

Calculations and Statistics

One-way ANOVA corrected by a post hoc analysis (Sidak) for multiple testing was used for testing differences between more than two groups. Unpaired two-sided t test was used to assess differences between two groups. $p < 0.05$ was considered significant. Calculations were made using GraphPad

Prism version 6.04 for Windows, GraphPad Software, <https://www.graphpad.com> and STAT14 (SE). For illustrations, the Adobe CC software suite was used. Data are shown as mean \pm SD. See [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.10.034>.

AUTHOR CONTRIBUTIONS

N.J.W.A. and J.J.H. conceptualized and designed of the study; C.Z.J., M.H., C.D., M.S., N.B.J., T.J., A.L., T.V., F.K.K., K.N.B.-M., T.I., B.F.-R., and S.M. provided clinical samples; N.J.W.A., R.E.K., L.S.G., M.N.G., E.B.-M., R.A., M.W.-S., K.D.G., and B.H. performed *in vitro*, *ex vivo*, and *in vivo* studies; N.J.W.A., D.H., F.M., and M.M. performed mass spectrometry-based proteomics; N.J.W.A., R.E.K., C.Z.J., M.H., D.H., C.D., M.S., L.S.G., N.B.J., M.N.G., E.B.-M., R.A., M.W.-S., K.D.G., F.M., T.J., A.L., T.V., F.F.K., K.N.B.-M., T.I., C.F.D., M.M.-R., B.H., B.F.-R., M.M., S.M., and J.J.H. and contributed to the analysis and interpretation of the data; N.J.W.A. drafted the manuscript; R.E.K., C.Z.J., M.H., D.H., C.D., M.S., L.S.G., N.B.J., M.N.G., E.B.-M., R.A., M.W.-S., K.D.G., F.M., T.J., A.L., T.V., F.F.K., K.N.B.-M., T.I., C.F.D., M.M.-R., B.H., B.F.-R., M.M., S.M., and J.J.H. critically revised the manuscript for important intellectual content. All authors have provided approval of the final version to be published. J.J.H. and N.J.W.A. are responsible for the integrity of the work as a whole.

ACKNOWLEDGMENTS

Mercodia A/S (Uppsala, Sweden), which kindly provided us with reagents for PG 1-61 ELISA, did not have any impact on the study, interpretation of results, or similar. None of the authors will obtain any financial benefits from Mercodia A/S regarding this publication. This work was supported by NNF Center for Basic Metabolic Research, University of Copenhagen, NNF application number: 13563 (Novo Nordisk Foundation, Denmark), EliteForsk Rejsestipendiat (2016), The Danish Council for Independent Research (DFF – 1333-00206A), Augustinus Foundation, Aase og Ejnar Danielsens Fond, Mærsk Fonden, Holger Rabitz fond, Læge Johannes Nicolaj Krogsgaard og hustru Else Krogsgaards minde-legat for medicinsk forskning og medicinske studenter ved Københavns Universitet, European Molecular Biology Organization (EMBO), and the European Foundation for the Study of Diabetes (EFSD).

Received: February 13, 2017

Revised: July 1, 2017

Accepted: October 5, 2017

Published: November 7, 2017

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